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16. ABSTRACT

Ponderosa Pine(Pinus ponderosa Laws.) seedlings grown in feild exposure chambers and fumigated with ozone at a concentration of 200 g/m^3 6 h/day; 7 days/wk for varible periods of up to 20 weeks. Pines were harvested at 4 wk intervals to determine the levels of the metabolites in tops (stem and needles) and roots. The ozone exposure affected the metabolite pools in the roots and tops differentially. The ozone treatment had no effect on the nitrogen or amino acid content of the tops but increased the levels of these pools in the roots. The levels of the soluble sugars, starch, and phenols tended to increase in the tops and decrease in the roots of the ozone-exposed plants. The ozone treatment had no effect on the lignin content in either the roots or the tops.

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Abstract. Ponderosa pine (*Pinus ponderosa* Laws.) seedlings were grown in field exposure chambers and fumigated with ozone at a concentration of 200 $\mu g/m^3$ 6 h/day; 7 days/wk for variable periods up to 20 weeks. Pines were harvested at 4 wk intervals to determine the levels of the metabolites in tops (stem and needles) and roots. The ozone exposure affected the metabolite pools in the roots and tops differentially. The ozone treatment had no effect on the nitrogen or amino acid content of the tops but increased the levels of these pools in the roots. The levels of soluble sugars, starch, and phenols tended to increase in the tops and decrease in the roots of the ozone-exposed plants. The ozone treatment had no effect on lignin content in either the roots or tops. **Forest Sci. 22:234–241.**

Additional key words. *Pinus ponderosa*, air pollution.

FOREST COMMUNITIES are being subjected to increasing amounts of air pollution. For example, oxidant injury has been identified on native white pine in several areas of the eastern and southeastern United States (Berry and Ripperton 1963). Oxidants transported from the Los Angeles basin have caused extensive injury in the surrounding forested areas (Richards and others 1968, Miller 1973) and oxidant injury has been observed in other parts of California (Miller and Millecan 1971). Studies have established that oxidant air pollutants, primarily ozone, are the cause of the chlorotic decline disease reported in the San Bernardino National Forest (Parmeter and Miller 1968, Richards and others 1968, Miller and others 1969). The disease is characterized by a chlorotic mottling of the needles, reduced needle growth and retention, progressive reduction in tree growth, decline of fibrous roots, and tree mortality (Parmeter and Miller 1968). The decline apparently results from a progressive reduction in the ability of the affected trees to meet their energy requirements (Parmeter and Miller 1968). The objective of this study was to determine if a low level of ozone could cause significant changes in the metabolite pools of ponderosa pine that could be associated with either the symptoms of the disease or to the reduced growth.

Materials and Methods

Plant Material. Ponderosa pine (*Pinus ponderosa* Laws.) were grown from a stratified (5°C for 4 wk) Oregon seed source and planted in 2.4 l pots containing a Jiffy Mix Plus[1] potting medium. The seedlings emerged within 2 wk of planting and were thinned to 4 plants per pot. The pots were placed in field exposure chambers (Heagle and others 1973) covered with

Plant Physiologist, Plant Pathologist, and Biologist, respectively, Environmental Protection Agency, 200 S.W. 35th Street, Corvallis, Oregon 97330. The assistance of Dr. L. Male, Environmental Protection Agency, Corvallis, Oregon, with statistical analysis and interpretation is appreciated. A portion of these data has previously been published (Tingey 1974) and is presented in the Figures for comparison purposes. Manuscript received July 8, 1975.

TABLE 1. Summary of ambient environmental conditions during ozone exposure.[1]

Weeks after initiation of exposure	Temperature, °C			Relative humidity, percent		
	Average maximum	Average minimum	Average median	Average maximum	Average minimum	Average median
4	27	15	21	69	25	47
8	28	15	22	72	23	48
12	23	11	17	87	30	59
16	18	9	13	96	44	70
20	14	9	12	95	62	79

[1] All temperature and relative humidity values are 4-week averages during the exposure hours (9 a.m.–3 p.m., PDT).

Saran[1] shade cloth (50 percent shade). Past experience (Heagle and others 1973) has shown that exposure chamber and ambient environmental regimen were very similar except during the hottest hours of the day when slightly elevated temperatures occurred in the exposure chambers. With this knowledge, ambient environmental data serve as a reliable estimate of exposure chamber conditions. Ambient temperature and relative humidity are summarized by 4-week intervals for the actual exposure hours (Table 1). The ozone exposures were initiated 1 wk after seeding and were maintained at either 0 or 200 $\mu g/m^3$ for 6 hr/day (9 A.M.–3 P.M., PDT) and continued for 20 wk. Ozone was produced by ultraviolet radiation of charcoal filtered air and measured with a chemiluminescent monitor.

Every fourth week after planting, three samples were harvested from each of three control and ozone chambers. A sample consisted of the plant material from 2 pots, each containing 4 plants. The plant tops (needles and stems) were removed, the growth medium was washed from the roots, and all plant material was oven dried at 70° C for 48 hr. The dried plant tops and roots were separately ground with a Wiley mill (40 mesh screen).

Metabolite Analysis. Samples were placed in pre-extracted cellulose thimbles and extracted for 4 hr with 80 percent ethanol in a microsoxhlet extractor. The extracts were cleared with lead acetate and bone ash, and the soluble sugars measured by the anthrone method using glucose as a standard (Ebell 1969a). The remaining residue after the 80 percent ethanol extraction was used for starch determination according to the glucose oxidase-iodide procedure (Ebell 1969b). For the analysis of amino acids, phenols, and lignin, the samples were placed in pre-extracted thimbles and extracted with 10 percent methanol in a microsoxhlet extractor for 3 hr. Aliquots of the extract were used to determine amino acids with valine as a standard (Yemm and Cocking 1955) and phenols were determined according to Anderson and Todd (1968) using chlorogenic acid as a standard. The residue remaining after the methanol extraction was refluxed with ethanol:benzene (2:1; V:V) for 1 hr, then extracted with water and 50 percent methanol. The residue, hydrolyzed with 0.5 N NaOH for 16 hr at 70° C (Jeffs and Northcote 1966), was analyzed for lignin content according to Stafford (1960). To determine Kjeldahl nitrogen, the samples were digested (Taras and others 1971) and the resulting ammonia was measured using the phenol-hypochlorite reaction (U.S. Environmental Protection Agency 1974).

Data Analysis. The principles of general linear models analysis were employed to formulate hypotheses and conduct statis-

[1] Mention of a product name does not imply the endorsement by the Environmental Protection Agency.

TABLE 2. Test of hypotheses for metabolite pools of ponderosa pine exposed to ozone.[1]

	Hypothesis	Metabolites					
		Kjeldahl N	Amino acids	Soluble sugars	Starch	Phenol	Lignin
I	Is there a difference among harvests for any combination of treatment × plant part?	**	**	**	**	**	**
II	Is there a difference between treatments for any combination of harvest × plant part?	+	+	**	**	**	NS
III	Is there a difference between plant parts for any combination of harvest × treatment?	*	**	**	**	**	**
IV	Are the differences between treatment means invariant to plant parts for each harvest?	**	**	**	**	**	**
V	Are the dilfferences between treatment means invariant to harvest for each type of plant part?	NS	**	**	**	**	NS
VI	Are the differences between plant part means invariant to harvests for each treatment?	NS	NS	**	**	**	**
VII	Are the differences among the treatment × plant parts mean invariant to harvests?	NS	NS	**	**	**	**

[1] Probability of rejection when hypothesis is true: + ≤10%; * ≤5%; ** ≤1%; NS = greater than 10%. Plant part refers to either tops (stem + needle) or roots.

tical tests (Searle 1971).[2] The hypotheses formed and the results of the testing are shown in Table 2. The statistical techniques used will not be discussed in detail but an example follows. Hypothesis VI (Table 2) means that the differences between root and top do not depend upon harvest date but they can depend upon ozone level. The hypothesis will be false (and probably rejected statistically) if for some level of ozone the differences between the top and root does depend upon harvest date. This hypothesis involves three factors and does not correspond to any of the traditionally used Analysis of Variance Tests. This hypothesis is meaningful, however, and the statistical procedures used for testing possess all the properties of standard Analysis of Variance Tests. In the discussion which follows, references will be made to both statistically significant differences and practical differences. In the presentation of results, all data presented exhibited both statistical and meaningful (practical) significant differences unless otherwise stated.

[2] Male, L. M. 1974. MSP (Model Specification Program)—an easy to use linear statistical analysis system. Proceedings of Automated Data Processing Workshop. Office of Research and Development. Environmental Protection Agency. Washington, DC.

Results

Foliar injury was observed on the cotyledonary needles after 12 weeks of exposure. At subsequent harvests additional

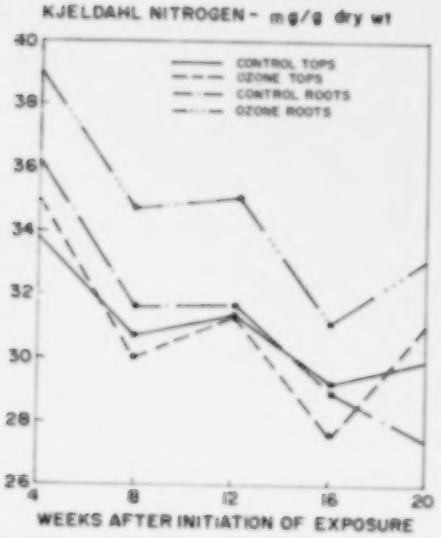

FIGURE 1. *Changes in Kjeldahl nitrogen as a function of plant part, seedling age, and ozone treatment. Means are based on 9 observations,* $s_{\bar{x}} = 1.3$.

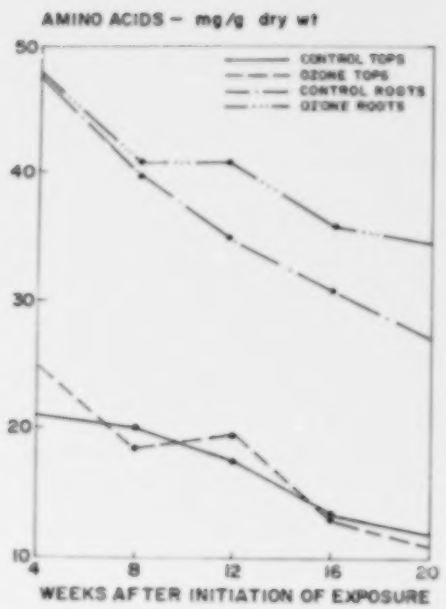

FIGURE 2. *The influence of ozone treatment, plant part, and seedling age on the amino acid content of pine seedlings. Means are based on 9 observations,* $s_{\bar{x}} = 1.95$.

foliar injury developed on the primary needles, but little injury developed on the secondary needles. It is emphasized that the foliage of the 1-year-old seedlings was dominated by primary needles. The presence of secondary needles was by exception and, when present, represented a small percentage of the foliage. There were no phenological differences between treatments.

Nitrogen Metabolism. Table 2 shows the significance levels of the various hypotheses tested concerning Kjeldahl nitrogen and amino acids. The Kjeldahl nitrogen content of both the roots and top of ponderosa pine seedlings declined markedly throughout the course of the experiment (Figure 1). This seasonal decline, similar in both roots and tops, was not influenced by the ozone treatment. The ozone treatment had no significant effect on the nitrogen content of the tops in the experiment. In contrast, the nitrogen content of the roots of the ponderosa pine seedlings exposed to ozone was significantly greater than the controls throughout the experiment.

The amino acid content of the tops and roots of the seedlings decreased significantly throughout the experiment (Figure 2). The amino acid content of the roots was always greater than that of the plant tops. The ozone treatment differentially affected the amino acid content of the roots and tops of the seedlings. The tops of the control and ozone-treated plants contained similar levels of amino acids in the experiment. However, significant levels of amino acids accumulated in the roots of the exposed plants. The accumulation was most apparent after visual injury occurred following 12 wk of exposure and remained higher than in the controls for the remainder of the experiment.

Carbohydrate Metabolism. Table 2 shows the significance levels of the hypotheses tested concerning the content of soluble sugars and starch in pine seedlings. The soluble sugars in the tops and roots tended to increase during the experiment except

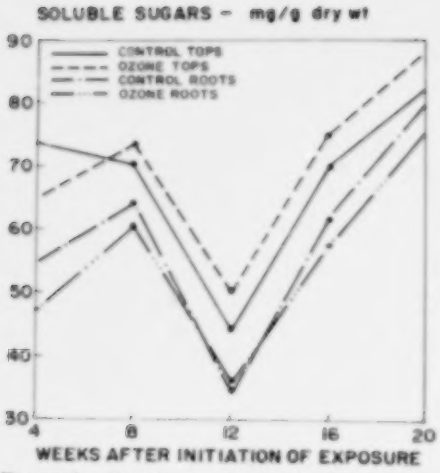

FIGURE 3. *The changes in soluble carbohydrates of ponderosa pine seedlings exposed to ozone throughout the growing season. Each mean is based on 9 observations,* $s_{\bar{x}} = 2.09$.

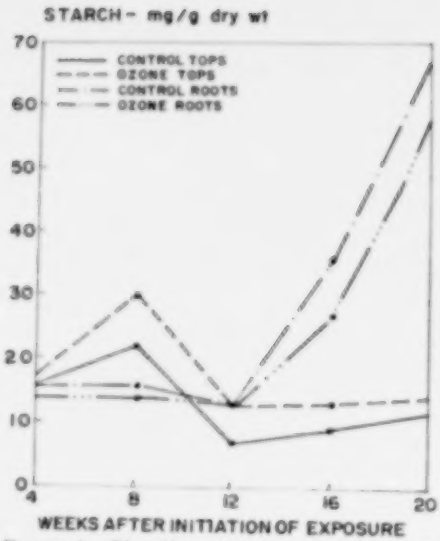

FIGURE 4. *The effects of ozone treatment on the levels of starch in the tops and roots of ponderosa pine seedlings. Each mean is based on 9 observations,* $s_{\bar{x}} = 1.83$.

at the third harvest, 12 wk of treatment (Figure 3). The ozone treatment differentially affected the accumulation of sugars in the tops and roots. Ozone exposure decreased the sugar content in the tops after 4 wk of exposure, but at the second and later harvest the tops of the ozone-exposed plants contained more soluble sugars than the controls. In contrast, the soluble sugars in the roots of the ozone-exposed plants were below the control level except after 12 wk of exposure when the levels were similar.

Early in the experiment, the starch concentration of the tops was similar to, or greater than, the level in the roots (Figure 4). After 12 wk into the experiment (mid-September), the starch levels in the roots were higher than in the tops. As with the soluble sugars, the ozone treatment affected the starch content of the tops and roots differently. In the tops, the starch level in the ozone-exposed plants exceeded the control level except at the first harvest where the amounts were similar. In contrast, the starch levels in the roots of the exposed and control plants were similar until 12 wk of exposure, when the control plants began accumulating starch at a greater rate than the exposed ones. By the end of the study, roots of the control plants contained about 15 percent more starch than the roots of the ozone-treated plants.

Phenol and Lignin Metabolism. The significance levels of the various hypotheses tested concerning phenol and lignin content are shown in Table 2. The phenol content of the roots and tops varied differentially during the experiment in response to seasonal changes and ozone treatment (Figure 5). The phenol content of the tops tended to increase throughout the growing season, while the phenol concentration in the roots was lower than that in the tops during most of the season. Phenols accumulated to higher levels in the tops of the exposed plants than in the tops of the controls. This accumulation was apparent after 8 wk of exposure and at the termination of the study, the exposed plants contained 13 percent more phenol than the control plants. In contrast, the phenol concentrations in the roots of the exposed plants were below the concentration in the control roots except after 12 wk of exposure.

At the start of the study, the lignin levels

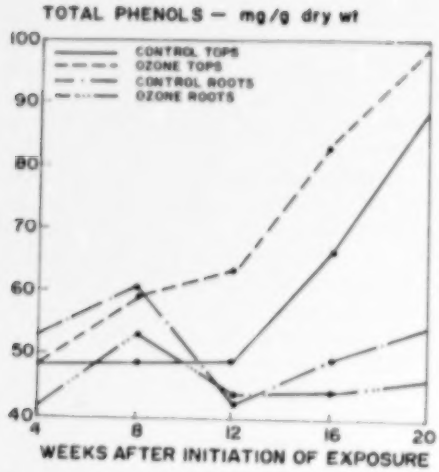

FIGURE 5. *The changes in the phenol content of the tops and roots of ponderosa pine seedlings as influenced by ozone. Each mean is based on 9 observations,* $s_{\bar{x}} = 2.67$.

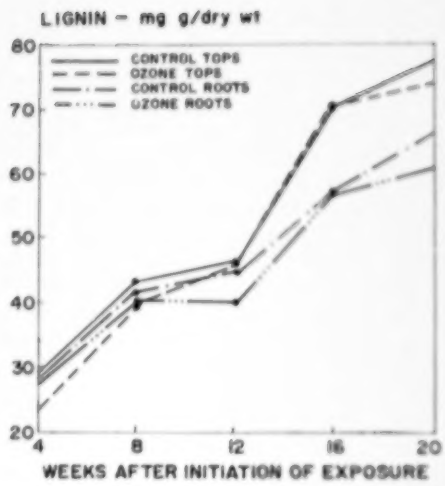

FIGURE 6. *The changes in the lignin content of ponderosa pine seedling roots and tops throughout the growing season. Each mean is based on 9 observations,* $s_{\bar{x}} = 2.13$.

were similar in the tops and roots, but as the plants matured, the lignin levels increased more rapidly in the tops than in the roots (Figure 6). The ozone exposure had no effect on the lignin content of either tops or roots.

Discussion

In previous studies in which herbaceous plants were exposed to acute ozone doses that caused visible foliar injury within 24 hr, there was a rise in amino acids (Tomlinson and Rich 1967, Ting and Mukerji 1971, Tingey and others 1973). This was attributed to either blocked protein synthesis or enhanced protein degradation (Tomlinson and Rich 1967, Ting and Mukerji 1971). In contrast, there was no increase in free amino acids or decrease in protein, as measured by Kjeldahl nitrogen, in the tops of ponderosa pine seedlings either before or after the appearance of visual injury. The lack of an ozone induced decrease in foliar protein level has been reported by others (Leone and others 1966, Adepipe and others 1973, Tingey and others 1973). As visible injury developed, the amino acids increased in the roots, possibly translocated from the injured tops. Amino acids are translocated from plant tops to roots where they are stored for subsequent mobilization to the tops (Kursanov 1963, Pate and Flinn 1973).

The decrease in soluble sugars observed after the first month of exposure could have resulted from a depression in the photosynthetic process. Several workers have shown that ozone will depress photosynthesis and induce stomatal closure (Hill and Littlefield 1969, Barnes 1972a). Miller and others (1969) reported that chronic ozone exposure depressed the photosynthetic rate of older needles, but stimulated the photosynthesis of current year needles. The depression in photosynthesis occurs prior to the appearance of visual injury (Miller and others 1969).

Chronic ozone exposures reportedly have reduced the concentrations of soluble sugars in 1-year-old needles while concentrations in current year needles were similar to the control (Miller and others 1969). Increased soluble sugars, observed in this study as a result of a chronic ozone exposure, have been reported previously (Barnes 1972b, Dugger and Palmer 1969). These authors suggested that the increased soluble sugars resulted from either a de-

crease in the rate of starch synthesis or an increase in starch hydrolysis. This conclusion seems inconsistent with the increase in starch observed in this study and the report by Hanson and Stewart (1970) showing that chronic levels of oxidant pollution hindered starch hydrolysis.

The increase in soluble sugars and starch in plant tops could have resulted from either stimulated photosynthesis, depressed respiration or reduced translocation of photosynthate. However, the reported effects of ozone on photosynthesis and respiration suggest that alterations of these processes are not responsible for the increase (Barnes 1972a, Miller and others 1969). The concurrent increase in sugars and phenols suggests that the retention of sugars in the tops resulted from the formation of glycosides that rendered the sugars unavailable for translocation to the roots or growth.

The accumulation of phenols in pine tops was similar to previously reported increases (Menser and Chaplin 1969, Howell and Kremer 1973, Howell 1974, Koukol and Dugger 1967). Howell (1974) has reported that the pigments in ozone injured tissue are phenolic in nature. This requires that either adequate phenol levels be present when the exposure occurs or that ozone stimulate phenol biosynthesis. Acute ozone exposures can stimulate enzymes of phenol metabolism (Tingey and others 1975), and phenols accumulate in the foliage before visual injury develops (Howell 1974). The increased phenols in pine tops preceeding visible injury suggests that elevated phenol levels are associated with injury development.

The concentration of 200 $\mu g/m^3$ ozone for 6 hr/day used in this study is well below the levels that occur in the San Bernardino area. There the levels exceed 200 $\mu g/m^3$ for 8 to 11 hr/day throughout the growing season (Miller 1973). This suggests that more severe changes would be expected to occur on the trees in that area because they are receiving a higher ozone dose than the one used in this study.

In summary, the effects of chronic ozone levels on ponderosa pine are: stimulation of respiration, depression or no significant effect on photosynthesis, and retention of soluble sugars and starch in the tops. These foliar changes are associated with a reduction of soluble sugars in the phloem (Miller and others 1968) and a reduction in soluble sugars and starch in roots. The changes in photosynthetic products are associated with a decline in root growth (Parmeter and Miller 1968). The observed reductions in root growth could be expected to cause a further decline in photosynthesis (Humphries and Thorne 1964). These observations suggest that the chlorotic decline disease could result from the tree's inability to meet its energy requirements. Also, the reduced level of storage metabolites in the roots and the reduced needle retention would decrease needed metabolic reserves required for growth (Kozlowski and Winget 1964).

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